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Kinetics of tris (1-chloro-2-propyl) phosphate (TCIPP) metabolism in human liver microsomes and serum

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Abstract

Tris(1-chloro-2-propyl) phosphate (TCIPP) is an emerging contaminant which is ubiquitous in the indoor and outdoor environment. Moreover, its presence in human body fluids and biota has been evidenced. Since no quantitative data exist on the biotransformation or stability of TCIPP in the human body, we performed an in vitro incubation of TCIPP with human liver microsomes (HLM) and human serum (HS). Two metabolites, namely bis(2-chloro-isopropyl) phosphate (BCIPP) and bis(1-chloro-2-propyl) 1-hydroxy-2-propyl phosphate (BCIPHIPP), were quantified in a kinetic study using HLM or HS (only BCIPP, the hydrolysis product) and LC-MS. The Michaelis-Menten model fitted best the NADPH-dependent formation of BCIPHIPP and BCIPP in HLM, with respective V_{MAX} of 154 ± 4 and 1470 ± 110 pmol/min/mg protein and respective apparent K_m of 80.2 ± 4.4 and 96.1 ± 14.5 μ M. Hydrolases, which are naturally present in HLM, were also involved in the production of BCIPP. A HS paraoxonase assay could not detect any BCIPP formation above 38.6 ± 10.8 pmol/min/ μ L serum. Our data indicate that BCIPP is the major metabolite of TCIPP formed in the liver. To our knowledge, this is the first quantitative assessment of the stability of TCIPP in tissues of humans or any other species. Further research is needed to confirm whether these biotransformation reactions are associated with a decrease or increase in toxicity.

Keywords

Organophosphate flame retardant; biotransformation; liver; serum; clearance

43 **Abbreviations**

44 BCIPHIPP: bis(1-chloroisopropyl) 1-hydroxyisopropyl phosphate

45 BCIPP: bis (1-chloroisopropyl) phosphate

46 HLM: human liver microsomes

47 PFR: phosphate flame retardant

48 QTOF: quadrupole-time-of-flight

49 TCIPP: tris(1-chloroisopropyl) phosphate

50 TRIS: tris (hydroxymethyl)aminomethane

51

1. Introduction

Tris(1-chloro-isopropyl) phosphate (TCIPP) is an additive flame retardant which is used in polymers, such as polyurethane foams, and is used also as a replacement of tris(2-chloroethyl) phosphate (EU 2008). TCIPP has been reported as one of the predominant phosphate flame retardants (PFRs) in the indoor and outdoor environment (van der Veen and de Boer 2012), and its uptake in living organisms has been demonstrated by several studies (Sundkvist et al. 2010, Greaves and Letcher 2014). In comparison to non-chlorinated PFRs, TCIPP could be more resistant to abiotic degradation processes (Kawagoshi et al. 2002, Meyer and Bester 2004). In the benthic food web, bioaccumulation has been suggested by Brandsma et al. (2015). Our group recently detected a suite of PFR metabolites including a hydroxylated metabolite of TCIPP in human urine (Van den Eede et al. 2015a). While the detection of PFR metabolites in humans does not necessarily imply that the parent compounds are bioaccumulative, it does imply that human exposure occurs. This is concerning, as little is known about the possible effects on human health of chronic exposure to PFRs like TCIPP. Atopic dermatitis was recently correlated with increasing dust levels of TCIPP in an epidemiological study (Araki et al. 2014). *In vitro*, agonistic activity towards the pregnane X receptor and increases in 17- β -estradiol and testosterone were noted as effects on the endocrine system (Kojima et al. 2013, Liu et al. 2012), yet no estrogenic effects were detected (Zhang et al. 2014). In animals, a possible interference with thyroid hormone axis for growth and development in chicken embryos by TCIPP was observed (Farhat et al. 2013), though in zebrafish limited neurobehavioral changes and no teratogenic or hormonal effects were reported for this flame retardant (Dishaw et al. 2014).

While biomonitoring can assist in correlating human health parameters with exposure to TCIPP, urinary analysis of the TCIPP metabolite, bis(2-chloro-isopropyl) phosphate (BCIPP), has had little success in contrast to other PFR metabolites which were targeted in

epidemiological studies (Schindler et al. 2009, Van den Eede et al. 2013a, Butt et al. 2014, Dodson et al. 2014, Fromme et al. 2014). Based on the results of *in vitro* metabolism screening (Van den Eede et al. 2013b) and a biomonitoring study (Van den Eede et al. 2015a) we suggested that another metabolite, namely bis(2-chloro-isopropyl) hydroxy-isopropyl phosphate (BCIPHIPP), might be a more suitable biomarker than BCIPP for monitoring exposure to TCIPP. However, a comparison based on quantitative data for both BCIPP and BCIPHIPP metabolites has not yet been made *in vitro*, nor *in vivo* to support this hypothesis.

Investigation of the production rates of these metabolites and their contribution to TCIPP clearance, and insight in the involved metabolic processes is a first step towards obtaining factors for the conversion of urinary concentrations to exposure estimates. Additionally, such toxicokinetic measurements are helpful in estimating the degree of persistence and the rate of (de)toxification of this flame retardant in the human body (depending on the role of the metabolites in the toxicity mechanism).

Liver is the major site of metabolism for many xenobiotics, where the majority of oxidative enzymes are expressed (Lipscomb and Poet 2008). Yet in the case of organophosphate triesters, hydrolytic enzymes such as paraoxonases could also be involved in biotransformation (Furlong et al. 2000). Paraoxonases, and more specifically PON1, have been identified as the responsible enzymes for detoxification of the toxic “oxon” metabolites of organophosphate pesticides, including diazoxon, chlorpyrifos-oxon, and paraoxon (Furlong et al. 2000). PON1 is expressed both in liver and in serum (Furlong et al. 2000) and catalyzes the hydrolysis reaction with formation of a dialkyl phosphate (diethyl phosphate for paraoxon) and an alcohol (4-nitrophenol for paraoxon). For TCIPP only the hepatic metabolism has been studied so far, either focusing on oxidative processes or not distinguishing between oxidative and hydrolytic reactions (EU 2008, Van den Eede et al. 2013b, Abdallah et al. 2015). In order to provide a more complete dataset for integration in

pharmacokinetic models, it is necessary to investigate the stability of TCIPP in presence of liver and serum enzymes, such as PON1, and to confirm the extent of contribution of oxidative and hydrolytic enzymes to TCIPP degradation *in vitro*.

There were five aims to our study: (i) to apply μ -LC-QTOF technology to screening TCIPP metabolites formed by human liver microsomes (HLM), (ii) to investigate metabolite clearance in HLM by quantifying the production rates of BCIPP and BCIPHIPP, (iii) to compare the intrinsic clearance of BCIPP to that of BCIPHIPP and relate both to *in vivo* findings, (iv) to extrapolate the intrinsic *in vitro* hepatic clearance to *in vivo* hepatic clearance, and (v) and to investigate the extent of hydrolysis of TCIPP to BCIPP by serum enzymes.

2. Materials and Methods

2.1. Materials and Reagents

Tris (hydroxymethyl) aminomethane (TRIS), β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), CaCl_2 , paraoxon, and diphenyl phosphate-d10 (DPHP-d10) were purchased from Sigma Aldrich (Bornem, Belgium). TCIPP standard was acquired from Pfalz & Bauer (Waterbury, USA). BCIPP, bis(2-chloroethyl) phosphate-d8 (BCEP-D8), tris(2-chloroethyl) phosphate-d12 (TCEP-D12), and BCIPHIPP were synthesized at the Max Planck Institute (Göttingen, Germany). TCIPP was a mixture of three isomers, namely tris(1-chloro-2-propyl) phosphate, bis(1-chloro-2-propyl) 1-chloropropyl phosphate, and bis(1-chloropropyl) 1-chloro-2-propyl phosphate. All other reference and internal standards were of analytical grade, BCIPHIPP was a mixture of 2 isomers as described elsewhere (Van den Eede et al. 2015a). HLM pools were purchased from Xenotech LLC, a 50-donor pool was used for the metabolite screening assay (lot 1210267) and a 200-donor pool for the metabolism kinetics study (lot 1210347, appendix B). Surplus serum samples collected in the frame of another study (registered at <http://clinicaltrials.gov/>

with number NCT01778868) were pooled for use. This study was approved by the Ethical Committee of the Antwerp University Hospital (Belgian Registry number B30020097009) and all participants provided their written informed consent. Serum from 15 patients was pooled and stored at -20°C until analysis. Acetonitrile (analytical grade) was obtained from Merck KgA Chemicals (Darmstadt, Germany) and ultrapure water (18.2 MΩ) from an ElgaLabWater water purification instrument (Saint Maurice, France).

2.2.Biotransformation assays

2.2.1. HLM screening assay

Reaction mixtures contained 50 mM TRIS buffer adjusted to pH 7.4 at 37 °C and 100 μM TCIPP in a total volume of 0.98 mL (final concentrations). 20 μl of NADPH (1 mM final concentration) was repeatedly added (every 30 min) to ensure continued CYP activity. After 120 min, reactions were quenched using 1 mL of ice-cold acetonitrile and by storing the tubes on ice. Either no HLM or no NADPH was added in the negative controls.

2.2.2. Preliminary experiments to establish steady state conditions

2.2.2.1.HLM

Preliminary experiments were run to establish the steady state conditions by monitoring the formation of BCIPHIPP metabolite. Firstly, the optimal protein concentration was established using concentrations of 0.05 mg/mL up to 1.0 mg/mL HLM (final concentration). Secondly, the optimal incubation time was established starting from 0 to 15 minutes. Details on the preliminary experiments can be found in supporting information.

2.2.2.2.Serum

Incubation mixtures consisted of 100 mM TRIS buffer (adjusted to pH 8.5 at 37 °C), 2 mM CaCl₂, and serum in a 0.5 to 3% concentration (v/v), in a final volume of 500 μl. Reactions were initiated by adding 100 μM of TCIPP or paraoxon (positive control) in acetonitrile (1% of total volume). After 10 min, reactions were stopped by adding 110 μl of 20 mM Na₂EDTA

and 60 µl of 10% acetic acid in water. Diphenyl phosphate-d10 (20 ng) was added to paraoxon samples, and 20 ng of BCEP-d8 was added to the TCIPP incubations. 200 µl of methanol was added to paraoxon samples, after which samples were vortexed and centrifuged. Supernatants were filtered before analysis. Serum samples incubated with TCIPP were extracted on Oasis WAX SPE columns, to enrich the extracts for BCIPP and BCEP-d8 based on a urine extraction protocol (Van den Eede et al. 2013a). Cartridges were conditioned with 2 mL methanol and 2 mL Milli Q water. After loading of the samples, cartridges were washed with 1 mL Milli Q water and BCIPP and BCEP-d8 were eluted with 2 mL of 5% NH₄OH in methanol. The eluate was collected and evaporated under a gentle N₂ stream until dryness. Extracts were reconstituted in 100 µl 15% methanol in water.

2.2.3. Kinetic metabolism study (HLM)

Initial rate conditions were selected based on BCIPHIPP formation, which was linear up to 0.25 mg/mL protein (HLM) and 7.5 min. Therefore these conditions were selected for conduct of our HLM kinetic metabolism study. Reaction mixtures contained 50 mM TRIS buffer adjusted to pH 7.4 at 37 °C, HLM (0.25 mg/mL final protein concentration) and 1 to 300 µM TCIPP in a total volume of 0.98 mL (final concentrations). For each substrate concentration, five replicates were prepared. All samples were pre-incubated in a shaking water bath at 37 °C for 5 min. The reaction was started by adding 20 µl of NADPH stock solution (1 mM, final concentration), and quenched after 5 min by 500 µl of ice-cold acetonitrile and storing the tubes on ice. A fixed amount of IS (250 ng TCEP-d12 and BCEP-d8) was added to each tube before centrifugation (10 min at 3,500 rpm). The supernatant was transferred to a new set of tubes, evaporated to 1 mL using nitrogen gas and filtered through a 0.45 µm nylon filter into HPLC glass vials. Characterization of PON activity in HLM under these conditions is described in supporting information

2.3. Quality control (QC)

For the HLM assay, NADPH negative control samples consisted of 50 mM TRIS buffer, HLM (0.25 mg/mL final protein concentration) and 1 to 300 μ M TCIPP (final concentrations) in a total volume of 1 mL (in triplicate). Enzyme negative control samples consisted of 50 mM TRIS buffer, 1 mM NADPH and 1 to 300 μ M TCIPP (final concentrations) in a total volume of 1 mL (three replicates for each substrate level). Blank samples (three replicates) consisted of 50 mM TRIS buffer and HLM (0.25 mg/mL final protein concentration) in a total volume of 0.98 mL.

Recovery of BCIPP and BCIPHIPP in HLM incubation mixtures, and the recovery of BCIPP in serum incubation mixtures were tested as described in the supporting information.

2.4. Instrumental analysis

2.4.1. Screening with μ -LC-QTOF

An Eksigent 200 μ LC was coupled to an ABSciex Triple-ToF 5600 for screening of the TCIPP metabolites in HLM samples. Analytes were separated on a Halo C18 column (50 x 0.5 mm, 2.7 μ m) with a mobile phase of 5 mM ammonium acetate in water (A) and acetonitrile (B). The following gradient was used: 10% B was held for 0.5 min, followed by a linear increase to 30% B in 1 min (0.5 min hold), and then to 95% B in 1 min (0.6 min hold) at a flow rate of 50 μ L/min. After each analysis the column was re-equilibrated at 10% B during 0.6 min. An electrospray ionization source was used with the following parameters: gas 1, gas 2, and curtain gas were set at 15, 40, and 30 L/min, respectively. A source temperature of 300 $^{\circ}$ C was used, and ion spray fragmentor voltage was set at 4500 (negative mode) or 5000 (positive mode). TOF range was set to acquire masses between 100.0000 and 1000.0000 Da with an accumulation time of 250 msec. MS/MS spectra were recorded in IDA (information dependent acquisition) mode, with fixed collision energy of 35 V.

2.4.2. Quantitative analysis

For the TCIPP kinetics studies in HLM and serum, extracts were analyzed on an Agilent 1290 LC coupled to a 6460 triple quadrupole MS. Phenylhexyl column (100 x 2.1 x 2.6 μ m, Phenomenex) was used for separation of the extracts. The mobile phase consisted of 5 mM ammonium acetate in water (A) and acetonitrile (B), with the following gradient conditions for HLM extracts: 10% B (0.5 min hold), increase to 30% B in 2.5 min (2 min hold), increase to 40% B in 5 min, followed by a sharp increase to 95% B (3.5 min hold), and equilibration at starting conditions for 3.5 min. A different gradient program was used for serum extracts: 5% B (0-2 min), 20% B (at 2.5 min), 30% B (4 min), 40% B (6 min), 95% B (11-11.5 min), and equilibration at starting conditions for 7 min.

Flow rate was 0.3 mL/min, temperature 40 °C, injection volume 1 μ L. The following MS parameters were used: gas temperature 325 °C, sheath gas heater 250 °C, gas flow 10 L/min, sheath gas flow 11 L/min, nebulizer pressure 35 psi, capillary and nozzle voltage 3500 and 0 V, respectively. MRM transitions can be found in Table A1.

2.5.Data analysis

2.5.1. Statistics

Linear and non-linear regression of the HLM and serum data was done using Graphpad Prism 5 (GraphPad software, Inc). For HLM data, the following models were compared: Michaelis-Menten (equation 1), Hill equation, and substrate inhibition (see SI). BCIPP concentrations in cofactor and enzyme negative control samples were also analyzed by linear regression. Selection of the most appropriate model was based on an F-test of the goodness of fit of each model. If the difference in fit between models was not significant ($p>0.05$), the simplest model was selected. More details can be found in Supplementary Information.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (\text{Equation 1, Michaelis-Menten model})$$

For the analysis of serum data, an F-test was used after regression to determine if the slope was significantly different from zero. To estimate the sensitivity of the serum assay, Cohen's d for minimal effect size (equation 2), was considered as the minimal net formation of BCIPP by serum enzymes.

$$\bar{x}_1 - \bar{x}_2 = d \times \sqrt{\frac{(n_1-1) \times s_1^2 + (n_2-1) \times s_2^2}{n_1 + n_2 - 2}} \quad (\text{Equation 2})$$

2.5.2. *In vitro-in vivo* extrapolation

Intrinsic clearance and extrapolation of *in vitro* data to *in vivo* data were calculated based on the following equations (Lipscomb and Poet 2008):

$$CL_{int,LM} = \frac{V_{max}}{K_m} \quad (\text{Equation 3})$$

$$CL_{int} = CL_{int,LM} \times \frac{mg_{microsomalprotein}}{gliver} \quad (\text{Equation 4})$$

$$CL_h = \frac{CL_{int} \times Q_H}{CL_{int} + Q_H} \quad (\text{Equation 5})$$

Equation 3 represents the intrinsic *in vitro* clearance $CL_{int,LM}$, equation 4 scales $CL_{int,LM}$ to the full liver, while equation 5 assumes the direct scaling approach as representative of *in vivo* hepatic clearance (Poulin et al. 2011), although this approach may overestimate the *in vivo* clearance in case of extensive plasma protein binding. In these equations, the following scaling factors were used: 34 mg/g microsomal proteins per g liver, 0.71 mL/min/g liver for hepatic clearance (Q_H) and a relative liver mass of 2.6 g liver/kg bodyweight (Lipscomb and Poet 2008).

3. Results

3.1. Micro-LC-QTOF

Four metabolites were found within 3 ppm mass error margin (table A3, figure 1): BCIPP (2 isomers), BCIPHIPP (2 isomers), a carboxyl-metabolite (2 isomers) and hydroxy-TCIPP (1

isomer), which were previously named BCIPP, TCIPP-M2, TCIPP-M1 and TCIPP-M3, respectively (Van den Eede et al. 2013b).

Isomers were not completely separated (figure A1), because the μ -LC gradient program was not fully optimized in this quick screening assay. MS/MS spectra (figure A2) confirmed the general structure of the metabolites, by showing the protonated phosphate, mono-ester and sometimes di-ester fragments in positive ionization mode. TCIPP-M1 indicated two additional rearrangements, namely one to a mono-ester with additional loss of formic acid to an ethenyl side chain (m/z 124.9978) and one to a lactone structure (m/z 152.9944).

3.2.Performance of kinetic HLM and serumassay

3.2.1. HLM

The method detection limit (MDL) in HLM samples was 10 ng/mL for BCIPP and 1.5 ng/mL for BCIPHIPP. Using the optimized initial rate conditions for the experiments, TCIPP-M1 and TCIPP-M3 were undetectable, probably because of slower formation rates so that they could only be detected in the 2h screening assay.

3.2.2. serum

BCIPP formation in the serum assay did not show any significant increase with increasing enzyme concentrations in incubation mixtures. At 20 μ M TCIPP, BCIPP levels in samples were not higher than in method blanks. At 100 μ M TCIPP, BCIPP levels in samples with serum, buffer, and CaCl_2 (cofactor) were not higher than in samples without serum enzymes. The slope of BCIPP formation versus enzyme concentration was not significantly different from 0 ($p = 0.27$). A careful estimation of the sensitivity of the serum assay (equation 1) indicated that BCIPP formation would be below 38.6 (SD 10.8) pmol/min/ μ L serum. Compared to the positive control, 4-nitrophenol formation from 100 μ M paraoxon was on average 88.3 (SD 5.6) pmol/min/ μ L serum.

3.3.Metabolism kinetics in HLM

BCIPP was detected in reaction mixtures and also in our negative control samples. A large variability in BCIPP concentrations in samples was observed due to its variable presence in negative control samples (20 to 40% of total concentration). While BCIPP concentrations versus substrate level followed a non-linear trend characterized by saturation in samples (figure 2), this trend was linear in negative controls (figure 3). After subtraction of negative control levels, Michaelis-Menten (equation 1) was the optimal model for non-linear regression of NADPH-dependent BCIPP formation with an associated V_{MAX} of 1470 ± 110 pmol/min/mg protein, and K_m of 96.1 ± 14.5 μ M.

Linear regression of BCIPP concentrations versus substrate concentration in enzyme and cofactor negative controls indicated good fit with R^2 values of >0.95 for both curves. Both slopes were significantly different from 0 and also significantly different from each other (p values all <0.01 , see Table A2 for more information), indicating involvement of hydrolases in HLM, though their reaction rates were not quantifiable due to variable BCIPP formation in enzyme negative control replicates. We tested paraoxonase activity in HLM to test the possibility of hydrolases being involved in the production of BCIPP. Paraoxon hydrolysis by HLM under the same conditions (using 100 μ M substrate) was 64.5 pmol (4-nitrophenol)/min/mg protein, which supports the possible involvement of hydrolases in HLM.

BCIPHIPP was formed solely by CYP- or other NADPH-dependent enzymes. When plotting reaction velocity versus substrate concentration, a typical Michaelis-Menten curve was obtained with V_{max} of 153.5 ± 4.0 pmol/min/mg protein and K_m of 80.2 ± 4.4 μ M (figure 2). Production rate of BCIPHIPP was nearly tenfold lower than that of BCIPP, yet BCIPHIPP concentrations showed less variability in reaction mixtures as this metabolite was not present in negative control samples. As a result, the kinetics model that was predicted for BCIPHIPP had a lower standard error for constants such as apparent K_m and V_{max} .

Based on the V_{\max} and K_m parameters, *in vitro* intrinsic clearance $CL_{\text{int,L,M}}$ (equation 3) was estimated to be 15.3 ± 2.6 and 1.91 ± 0.12 $\mu\text{L}/\text{min}/\text{mg}$ protein for BCIPP and BCIPHIPP, respectively. As pointed out elsewhere (Pelkonen and Turpeinen 2007), $CL_{\text{int,L,M}}$ of different pathways can be summed resulting in 17.2 ± 2.6 $\mu\text{L}/\text{min}/\text{mg}$ protein. After application of equation 4 and the scaling factor mentioned under the methods section, the intrinsic clearance would be 585 $\mu\text{L}/\text{min}/\text{g}$ liver for TCIPP. Using the direct scaling approach (equation 5) this would be comparable to 0.32 $\text{mL}/\text{min}/\text{g}$ liver or 0.83 $\text{mL}/\text{min}/\text{kg}$ bodyweight..

4. Discussion

4.1. Micro-LC-QTOF screening

The qualitative profile of metabolites, namely BCIPHIPP as major metabolite, was comparable both to our previously published work (Van den Eede et al. 2013b) and to the findings of Abdallah et al. (2015). Although the latter did not report any presence of TCIPP-M3, this could have been due to the lower substrate concentrations used as TCIPP-M3 gave only a minor signal in our samples. Aside from demonstrating the reproducibility of our previous findings, the major purpose of repeating the metabolite screening for TCIPP was to test the applicability of $\mu\text{-LC-QTOF}$ in rapid analysis of suspect compounds. With a low injection volume, and only a three minute gradient elution program we were able to detect the same metabolites with a slightly better mass accuracy compared to the HPLC-QTOF instrument conditions in our previous study (Van den Eede et al. 2013b). While Abdallah et al. (2015) also achieved rapid separation with an ultra-high performance-LC coupled to an Orbitrap instrument, a $\mu\text{-LC-QTOF}$ is less expensive in acquisition and maintenance. The application of $\mu\text{-LC-QTOF}$ to this small sample set can serve as an indication of how similar sensitivity and resolution can be achieved along with a reduction in analysis time and solvent consumption in screening for metabolites of environmental contaminants.

4.2. Hydrolysis by serum enzymes

Although it did not seem likely that serum had a major influence on TCIPP metabolism, the sensitivity of our assay could be limited. Because of the low number of replicates, a lower standard deviation in the samples and negative controls were needed to distinguish a small catalytic effect of serum enzymes. Hence the sensitivity threshold may have interfered with the detection of any BCIPP formation and we cannot exclude any extra-hepatic degradation of TCIPP in blood. On the other hand, paraoxon hydrolysis by serum enzymes was observed, therefore the assay in itself was valid. This obvious difference between the catalytic activity of the paraoxonase enzyme towards paraoxon and TCIPP may be explained by structure-related differences, such as the absence of an aryl side chain, since paraoxonases are known to hydrolyze mainly dialkyl aryl phosphate structures (Testa and Krämer 2010). In spite of this lack of interaction between TCIPP and paraoxonases, we chose to test this enzyme family since to our knowledge, no other enzymes have been characterized in humans that are capable of hydrolyzing organophosphate triesters without being consumed in the reaction.

4.3. Biotransformation of TCIPP in HLM

The possible involvement of hydrolases in HLM in the formation of BCIPP is surprising considering the non-detectable BCIPP formation by serum enzymes. These contradictory results can have two explanations: either hydrolases with different characteristics or origin than paraoxonases are involved in TCIPP metabolism, or the serum assay did not achieve sufficient sensitivity to distinguish any effect of paraoxonases. As mentioned previously, degradation of TCIPP by serum enzymes cannot be ruled out.

As for the extrapolation of the kinetics of TCIPP biotransformation in HLM to the whole liver, the estimated intrinsic *in vivo* clearance of TCIPP (0.585 mL/min/g liver) is more than 80% of the average hepatic blood flow (section 2.5.2), suggesting that TCIPP is not a low clearance chemical (meaning intrinsic clearance <20% of hepatic blood flow) and that its

hepatic clearance is not flow-limited either. As a consequence, TCIPP is not likely to exhibit the same accumulating behavior in the human body as more apolar environmental contaminants, such as polychlorinated biphenyls or polybrominated diphenyl ethers (Darnerud et al. 2015).

4.4. Comparison of findings to *in vivo* toxicokinetics in rat

Exposure of rats to ¹⁴C-labeled TCIPP revealed quick absorption and distribution of TCIPP in the body, as after 5.7 h maximum concentrations of the radiolabel were found in the tissues (Minegishi et al. 1988). TCIPP was mostly found in the liver and the kidneys, followed by the lungs. The majority of the TCIPP radiolabel was excreted in urine (67% within one week) (Minegishi et al. 1988). No metabolite structures were identified or measured for comparison with clearance rates in this study, though its findings suggest that uptake of TCIPP in the liver occurs, which is a prerequisite for hepatic clearance. Therefore hepatic clearance could play an important role in elimination of TCIPP besides excretion.

4.5. Comparison of findings to human biomonitoring data

4.5.1. Human milk

The hepatic clearance rates measured in this study were comparable to those of TBOEP (Van den Eede et al. 2015b). However, in pooled breast milk samples higher detection frequency and median levels of TCIPP were reported in comparison to TBOEP (Sundkvist et al. 2010), which could be explained by high tissue distribution, as observed in rats for TCIPP (Minegishi et al. 1988). Investigation of toxicokinetic processes, such as renal and biliary clearance, and tissue distribution in the human body, is required to confirm the accumulation potential of this FR.

4.5.2. Human urine

TCIPP was detected only in a minority ($\leq 30\%$) of urine samples in several studies as mentioned in the introduction. While limited method sensitivity could have been a reason for

this observation in some cases, in others BCIPP was still rarely detected despite a lower method detection limit (Schindler et al. 2009, Dodson et al. 2014, Fromme et al. 2014). Another logical explanation could have been low exposure, which might be the case for the United States (Stapleton et al. 2009, Dodson et al. 2012). However, in Europe this scenario would be less likely since TCIPP has been reported as a predominant PFR in indoor dust and air (Fromme et al. 2014, Cequier et al. 2015, Brandsma et al. 2014). We did find BCIPHIPP recently in more than 90% of analyzed Australian urine samples at levels up to 9.4 ng/mL (Van den Eede et al. 2015a) even though dust levels of TCIPP in Australia were similar to those in Canada and the European mainland (Brommer, 2014). In light of the current findings, it seems that BCIPP is the major metabolite formed by liver enzymes, though two factors could explain the difficulty of detecting BCIPP in urine: (i) analytical difficulties, and (ii) possible pharmacokinetic processes interfering with its excretion in urine, such as protein binding, storage in tissues or other excretion pathways. Paired measurements of BCIPHIPP and BCIPP levels in urine and serum are needed to confirm this hypothesis.

4.6.Role of biotransformation in toxicity of TCIPP

To date, the toxicity of TCIPP and BCIPP or BCIPHIPP cannot be compared due to lack of data on these two metabolites. The biotransformation pathway to BCIPHIPP includes two intermediate structures (Abdallah et al. 2015) which could also exert toxic effects at the site of the liver. Consequently, we cannot state if the obtained clearance values represent a detoxification or bioactivation. Furthermore, the hydrolytic formation of BCIPP could produce 1-chloro-2-propanol as a byproduct. This chemical caused adverse effects on the liver in rats at doses of 100 mg/kg/day and higher during a period of fourteen weeks (NTP 1998), which is slightly higher than the low observed adverse effect level for TCIPP under similar conditions and toxicity endpoint (EU 2008). As far as the potential byproduct is concerned, hydrolysis of TCIPP would lead to a slight decrease in toxicity.

4.7.Limitations

The major limitations of this study as mentioned above, are the absence of plasma protein binding data, which could result in an overestimation of the current value of hepatic clearance; and the limited ability of the serum assay to distinguish any BCIPP formation.

Because not all four metabolites of TCIPP were detectable at the initial rate conditions in the HLM assay, our calculations were based only on the two major metabolites. This exclusion of the two other, but minor, metabolites may have impacted our estimation of the intrinsic clearance of TCIPP, leading to an underestimation.

5. Conclusions

This study is the first to present quantitative data on TCIPP metabolism in humans (or any other species). Our results indicated faster formation of the hydrolysis product BCIPP than the dechlorinated metabolite BCIPHIPP by hepatic enzymes. The role of serum hydrolases in TCIPP hydrolysis could not be confirmed, although hepatic hydrolases did contribute to BCIPP formation. More information is needed on the roles of the studied metabolites BCIPP and BCIPHIPP in the toxicity of this flame retardant.

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Supplementary material

Information regarding preliminary experiments, non-linear regression, and μ -LC-QTOF chromatograms and spectra are presented in appendix A.

CYP isoform activities in the commercially obtained HLM are available from appendix B.

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Figures

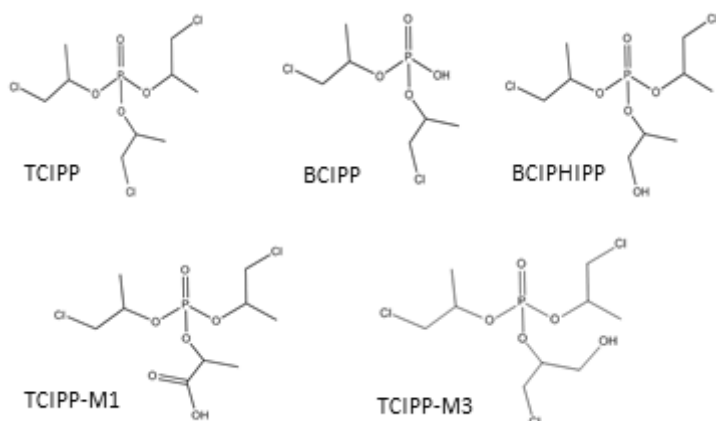


Figure 1. Proposed structures of tris(1-chloro-2-propyl) phosphate (TCIPP) metabolites. BCIPP: bis(1-chloro-2-propyl) phosphate; BCIPHIPP: bis(1-chloro-2-propyl)1-hydroxy-2-propyl phosphate; TCIPP-M1 and TCIPP-M3 were named according to the previous publication (Van den Eede et al. 2013b). Only the structures of BCIPP and BCIPHIPP (2nd isomer) were confirmed using nuclear magnetic resonance spectroscopy and authentic standards.

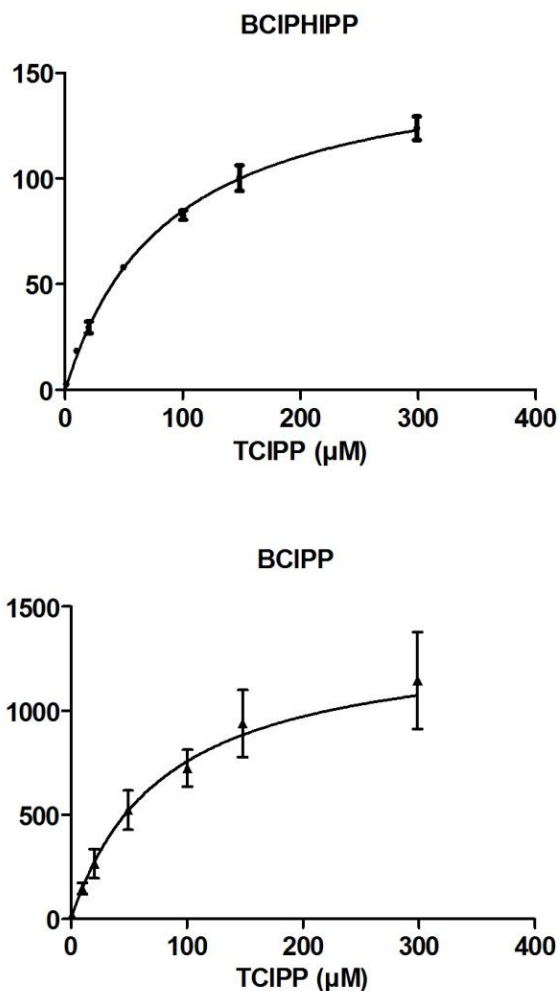


Figure 2. Formation of BCIPP (bis(1-chloro-2-propyl) phosphate; bottom panel) and BCIPHIPP (bis(1-chloro-2-propyl) 1-hydroxy-2-propyl phosphate; top panel) by NADPH-dependent enzymes. TCIPP (tris(1-chloro-2-propyl) phosphate) was incubated in the presence of 0.25 mg/ml human liver microsomes and 1 mM NADPH for 7.5 min at 37 °C. Y-axis: Metabolite formation rate (pmol/(min * mg microsomal protein)). Triangles indicate average production rate (n = 5), while error bars indicate the standard deviation between replicas.

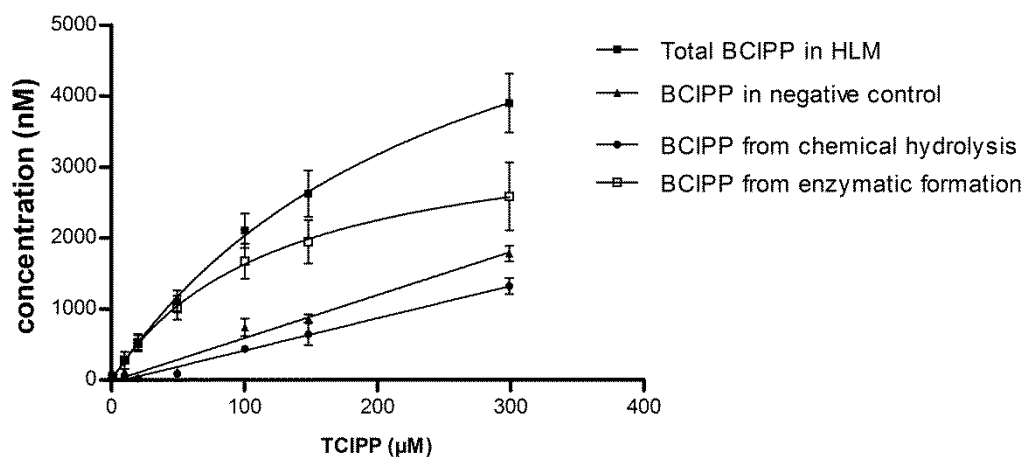


Figure 3. Comparison of BCIPP (bis(1-chloro-2-propyl) phosphate) levels in incubation samples of TCIPP (tris(1-chloro-2-propyl) phosphate) with human liver microsomes and/or buffer. Total BCIPP: BCIPP levels as a result of both enzymatic formation and chemical hydrolysis; BCIPP from enzymatic formation: sum of BCIPP due to NADPH-catalyzed reactions and due to enzymatic hydrolysis; BCIPP in negative control: chemical and enzymatic hydrolysis of TCIPP in absence of NADPH; chemical hydrolysis: BCIPP formation in presence of buffer alone. Symbols indicate average concentrations, error bars represent the standard deviation..

